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# Spatiotemporal phosphoregulation of Lgl: finding meaning in multiple on/off buttons

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## Abstract

Intracellular asymmetries, often termed cell polarity, determine how cells organize and divide to ultimately control cell fate and shape animal tissues. The tumor suppressor Lethal giant larvae (Lgl) functions at the core of the evolutionarily conserved cell polarity machinery that controls apico-basal polarization. This function relies on its restricted basolateral localization via phosphorylation by aPKC. Here, we summarize the spatial and temporal control of Lgl during the cell cycle, highlighting two ideas that emerged from our recent findings: 1) Aurora A directly phosphorylates Lgl during symmetric division to couple reorganization of epithelial polarity with the cell cycle; 2) Phosphorylation of Lgl within three conserved serines controls its localization and function in a site-specific manner. Considering the importance of phosphorylation to regulate the concentration of Lgl at the plasma membrane, we will further discuss how it may work as an on-off switch for the interaction with cortical binding partners, with implications on epithelial polarization and spindle orientation.

## Keywords

Epithelial tissue, cell-cycle, spindle orientation, Lgl, *Drosophila*, cell polarity, basolateral, aPKC, Dlg, phosphorylation

The epithelial tissue forms a critical barrier that protects animal organs from the external environment, providing mechanical support and controlling transport and signaling in a polarized manner. Epithelial polarity relies on the asymmetric distribution of cortical protein complexes to define the position of specialized cell junctions, and to orchestrate polarized vesicle trafficking and cytoskeleton organization along the apico-basal axis<sup>1</sup>. Common localization and genetic interactions placed the basolateral proteins Lethal giant larvae (Lgl), Discs large (Dlg) and Scrib in the same pathway that counteracts the activity of apical aPKC and Crumbs polarity complexes<sup>2,3,4</sup>. However, how these proteins interact to collaborate as basolateral determinants is still poorly understood. Furthermore, these proteins regulate other pathways that control cell proliferation, migration and cancer<sup>5</sup>. In fact, *lgl* was one of the first tumor suppressors identified and its human paralogues (Hugl-1 and Hugl-2) are now strongly linked to the etiology of cancer<sup>6-8</sup>. Lgl is regulated in a cell-cycle dependent manner as part of the mechanism that produces daughter cells with distinct fates during asymmetric cell division<sup>9</sup>. We and other group have recently discovered that phosphoregulation of Lgl is also required during symmetric division in *Drosophila* epithelial cells, where the cortical release of Lgl promotes planar spindle orientation<sup>10,11</sup>. In this commentary, we will discuss how phosphorylation of Lgl acts as a mechanism to temporally and spatially control its localization, interactions and activity.

### **Lgl interactions at the plasma membrane and the underlying cortex**

Early *Drosophila* studies documented Lgl localization in the cytoplasm or in association with the plasma membrane and the underlying actomyosin cortex<sup>12</sup>. Biochemical assays aiming to address the molecular basis for the cortical localization of Lgl revealed a physical interaction with non-muscle myosin II heavy chain<sup>13,14</sup>, which was also validated in mammalian cells<sup>15,16</sup>. However, Lgl recruitment to the cytoskeleton fraction was found to be partially independent of myosin II<sup>14</sup> and, accordingly, other cortical polarity proteins such as aPKC, Dlg and Scrib may form alternative complexes with Lgl<sup>9,17-20</sup>.

aPKC phosphorylation in three evolutionarily conserved residues (S656, S660 and S664 in *Drosophila*) disrupts the localization of Lgl at the cortex and the plasma membrane<sup>9,17</sup>. The combination of *in vitro* studies with the analysis of deletion mutants in *Drosophila* neuroblasts suggested that Lgl phosphorylation induces an intramolecular association between the C- and N-terminus, which masks the domains that mediate cortical and plasma

membrane association<sup>14</sup>. However, structural evidences of Lgl conformational changes are still lacking. A comparison with the crystallographic structure of its yeast homologue, Sro7, locates the phosphorylation sites within a flexible loop between two WD40 repeats of Lgl's two- $\beta$ -propeller fold<sup>21, 22</sup>. Importantly, this region is exposed at the surface, forming a platform for electrostatic interactions that provide an alternative mechanism to regulate the Lgl localization, independently of conformational changes. Recent work revealed that the phosphorylation sites are part of a positively charged basic and hydrophobic (BH) motif (Fig. 1A), which interacts with negatively charged plasma membrane phosphoinositides<sup>22, 23</sup>. Consistent with this, mutating the basic aminoacids of the BH domain dramatically disrupts Lgl localization and function. Phosphorylation can therefore control plasma membrane localization by rapidly altering the bulk electrostatic charge of the BH domain, thereby weakening the electrostatic attraction to phospholipids<sup>22, 23</sup>. Similar electrostatic control of the interaction of this domain with a negatively charged domain in non-muscle myosin II has also been proposed<sup>15, 16</sup>. Nevertheless, depletion of plasma membrane phosphoinositides by hypoxia induces full cortical release of Lgl, without affecting other cortical partners of Lgl, such as Myosin and Dlg<sup>22</sup>. Thus, Lgl interactions with other cortical proteins possibly require prior concentration of Lgl near the plasma membrane.

### **Spatiotemporal regulation of Lgl**

aPKC phosphorylates Lgl in epithelial cells during interphase to restrict its localization to the basolateral side<sup>9, 24</sup> (Fig. 1B). Additionally, this phosphorylation allows the polarized segregation of cell fate determinants during the asymmetric division of *Drosophila* neuroblasts and sensory organ precursor (SOP) cells<sup>9, 25</sup>. Lgl phosphorylation and cortical release breaks its inhibitory effect over aPKC and simultaneously allows association of Bazooka with Par-6/aPKC to induce phosphorylation of the cell fate determinants Numb and Miranda<sup>25, 26</sup>. It has been proposed that formation of the aPKC/Par-6/Baz complex is induced by Aurora A (AurA) - mediated phosphorylation of Par-6 and consequent activation of aPKC at mitotic entry<sup>25</sup>. However, a planar asymmetry of these proteins is firstly set during interphase in SOP cells<sup>27</sup>.

The epithelial tissue is in a constant balance between cell death and proliferation to control development and to maintain homeostasis<sup>28</sup>. How epithelial cells maintain overall tissue integrity during division is still largely unknown. Live imaging in *Drosophila* intact tissue recently provided significant insight into the reorganization of polarity complexes, cell

junctions and the cytoskeleton during epithelial cell division<sup>10, 11, 29-35</sup>. The cortical-release of Lgl at mitotic entry represents a striking observation since it differs radically from its known basolateral partners Dlg and Scrib<sup>10, 11, 36</sup>. Intriguingly, release of Lgl from the basolateral domain occurs in *aPKC* mutant epithelial cells, revealing the involvement of another kinase. The combination of *in vitro* kinase assays with *in vivo* genetic and drug inactivation led to the conclusion that AurA directly controls the release of basolateral Lgl in follicular and wing disc epithelia during mitosis via its ability to phosphorylate Ser656 and Ser664<sup>10, 11</sup> (Fig. 1B). AurA activity is critical to ensure Lgl mitotic release in epithelia since apical aPKC is physically separated from basolateral Lgl at mitotic entry. However, using unpolarized S2 cells, we also demonstrated that Lgl release is a general mitotic event, strongly delayed upon disruption of AurA activity<sup>11</sup>. Furthermore, Bell et al. proposed that Aurora A can directly remove Lgl from the entire cortex of larval neuroblasts during asymmetric cell division, whereas aPKC activation is only involved in the release of apical Lgl<sup>10</sup>. Thus, activation of the mitotic kinase AurA provides cell cycle control to the spatial regulation exerted by aPKC, maintaining Lgl localization under tight regulation during both symmetric and asymmetric division. Partial redundancy between the two kinases may ensure the robustness of mitotic events where the activity of AurA and aPKC partly overlaps.

### Site-specific phosphoregulation of Lgl

Both the conformational and electrostatic models imply the existence of two distinct pools of Lgl: an active non-phosphorylated fraction able to interact with actomyosin and the plasma membrane, and an inactive phosphorylated form that should remain in the cytoplasm. But what is the impact of phosphorylation in multiple serine residues? Are these phosphorylation sites equally important for the function, interaction with binding partners and plasma membrane localization? Our analysis of Lgl site-specific phosphoregulation brings out the significance of phosphorylation number and the site-specific importance of each phosphorylation to positively control the activity of Lgl.

Consistent with the importance of phosphorylation to induce cortical release of Lgl, mutation of the three phosphorylatable serines to alanines (Lgl<sup>3A</sup>) induces extension to the apical domain in *Drosophila* and mammalian epithelial cells and full cortical retention during epithelial and S2 cell mitosis<sup>10, 11, 19, 24, 37</sup>. Based on the finding that all double

mutants (Lgl<sup>S656A,S664A(ASA)</sup>; Lgl<sup>S656A,LglS660A(AAS)</sup>; Lgl<sup>S660A, S664A(SAA)</sup>) show significant cortical localization during mitosis, we have proposed that at least two phosphorylations are necessary for efficient Lgl cortical exclusion<sup>11</sup>. Another study reached similar conclusions analyzing the localization of equivalent Lgl double mutants upon overexpression of aPKC in S2 cells during interphase<sup>38</sup>. Following the recent work of the Prehoda and Hong labs<sup>22,23</sup>, one possible explanation is that two phosphorylations are required to switch the bulk electrostatic charge of Lgl's BH motif, fully repelling the interaction with phosphoinositides at the plasma membrane and therefore also blocking cortical localization (Fig. 2). Mutations in any combination of two of the three serines also prevent Lgl cortical exclusion during epithelial mitosis<sup>11</sup>, supporting the importance of multisite phosphorylation during cell division. However, in interphase, single phosphorylatable mutants display distinct abilities to support epithelial polarity or to exert dominant overexpression effects, highlighting the importance of site-specific phosphorylation for Lgl activity (Fig. 3)<sup>11</sup>. The presence of phosphorylatable S660 or S664 is sufficient for proper Lgl localization and activity. Furthermore, despite the apical localization of Lgl<sup>3A</sup> and Lgl<sup>SAA</sup>, only Lgl<sup>AAS</sup> has ability to induce loss of apical aPKC when strongly overexpressed, causing a dominant disruption of epithelial polarity<sup>11, 24</sup>. Thus, phosphorylation of Lgl on S664 seems to be of major importance for the positive regulation of Lgl as a basolateral factor. We will discuss several factors that may account for the phenotypes of mutants phosphorylatable in a single serine. Recent *in vitro* analysis of aPKC phosphorylation indicated that the three phosphorylation sites are not kinetically equivalent<sup>38</sup>. However, the kinetic parameters of singly phosphorylatable peptides expose a preferential order for phosphorylation (S664>S656>S660) that is insufficient on its own to explain the more inefficient apical exclusion of Lgl<sup>SAA</sup>. Nevertheless, the kinetics of aPKC phosphorylation may differ *in vivo*, where the catalytic activity of aPKC depends on multiple other factors including its interaction with Par-6<sup>39,40</sup>. Moreover, each phosphorylation may have a different impact on the release from the plasma membrane and myosin. Accordingly, singly phosphorylatable mutants have distinct localization during S2 cell mitosis, where Lgl<sup>SAA</sup> shows much stronger cortical retention (Fig. 3). Thus, regardless of the redundant ability of aPKC and AurA to phosphorylate S656 during S2 cell mitosis, this phosphorylation alone has the weakest effect on the dissociation from myosin and plasma membrane.

It is also unclear how some singly phosphorylatable mutants can be completely removed from the apical cortex (Fig. 3), whereas two phosphorylations are required for efficient cortical exclusion during mitosis. Phosphorylation-dependent interactions that anchor Lgl at the basolateral cortex of epithelia would reconcile these observations. Dlg could provide these interactions since aPKC phosphorylation in any of the three residues of human Lgl2 induces binding to the Guanylate kinase (GUK) domain of Dlg4 *in vitro*<sup>19</sup> (Fig. 2). Thus, double mutants should bind Dlg's GUK domain in the follicular epithelium through the available phosphorylated serine. However, our study would be consistent with the possibility that each phosphorylated serine confers distinct abilities to bind Dlg and to counteract the activity of apical proteins in the following order: Ser664>Ser660>Ser656 (Fig. 3)<sup>11</sup>. Dissociation from myosin and plasma membrane may increase the pool of Lgl available to interact with Dlg, or other cortical proteins, in order to control epithelial polarity (Fig. 2). Thus, the preferential phosphorylation by aPKC on S664<sup>38</sup> allied to its ability to lower the interaction with the plasma membrane provides one possible explanation for the higher activity of Lgl singly phosphorylated on S664.

### **Significance of Lgl phosphoregulation during epithelial mitosis**

Lgl cytoplasmic relocation at mitotic entry raises the possibility of a general function to promote faithful chromosome segregation. This is consistent with an early study performed in mammalian HEK293 cells that described mitotic spindle misorganization and chromosome missegregation upon overexpression of Lgl2 C-terminal domain or upon Lgl1 and Lgl2 knockdown<sup>41</sup>. A similar mitotic role for Lgl has also been documented in the wing imaginal disk epithelium<sup>10</sup>. However, the relevance of Lgl on chromosome segregation seems to be context-specific as we did not detect defects that could be unambiguously linked to chromosome segregation upon loss of *lgl* function in syncytial embryos, follicle cells and S2 cells<sup>11</sup>.

Lgl cortical displacement could also be involved in the control of cortical-dependent events during mitosis since, for instance, the reorganization of the actomyosin cortex supports a number of important functions, including mitotic cell rounding, spindle orientation and cytokinesis<sup>42</sup>. Follicle cells expressing the aforementioned Lgl double mutant forms or a membrane-targeted form of Lgl revealed defects in spindle orientation axis<sup>10,11</sup>. So, which cortical mechanisms controlling mitotic spindle orientation could be potentiated by Lgl cortical release? Lgl binding to non-muscle myosin II (NMII) heavy chain

has been proposed to inhibit Myosin filament formation *in vitro*<sup>15, 16</sup>. Thus, it would be reasonable to consider that Lgl exclusion induces changes in cortical actomyosin contractility and possibly in mitotic cell rounding, which are known to influence planar spindle alignment<sup>34, 43</sup>. However, despite the cortical retention of Lgl<sup>3A</sup> during mitosis, and possible inhibition of actomyosin contractility, Lgl<sup>3A</sup> expression does not affect mitotic spindle orientation<sup>11</sup>.

The main difference between Lgl<sup>3A</sup> and the double mutant forms could lie in the ability to bind Dlg's GUK domain<sup>19</sup>. Dlg participates in the planar orientation of cell division in epithelia, acting on the recruitment of the spindle orientation protein Pins (LGN in vertebrates), which mediates the connection of astral microtubules to the lateral cortex<sup>34, 44, 45</sup>. Pins interaction with Dlg is also controlled by Aurora A phosphorylation<sup>46</sup>. Furthermore, functional and crystallographic studies have shown that phosphorylated Pins and Lgl bind to the same region of Dlg's domain<sup>19, 45, 47, 48</sup>. Thus, maintenance of the Lgl/Dlg complex during mitosis is anticipated to impair Pins ability to bind Dlg, with the consequent spindle orientation defects that are observed upon expression of double mutant forms of Lgl in the follicular and wing disc epithelia<sup>10, 11</sup>. Importantly, the Pins pathway is activated during prophase<sup>46</sup>, concurrent with Lgl cortical release<sup>11</sup>. Aurora A may therefore coordinate the dissociation of the Lgl/Dlg complex with the formation of Pins/Dlg complex. However, it is unclear why all Lgl is released from the cortex and the plasma membrane to transiently free Dlg. The finding that membrane-targeted Lgl induces spindle orientation defects<sup>10</sup> favors the importance of decreasing Lgl local concentration, which would compete with phospho-Pins despite the lower affinity of phospho-Lgl to the Dlg's GUK domain<sup>19, 47</sup>.

### Conclusions and perspectives

Recent advances highlight the importance of cell cycle dependent kinases for the regulation of polarity proteins to reshape the organization of the cytoskeleton during epithelial mitosis, and for the preservation of tissue architecture during proliferation. Aurora A provides the link between cell cycle regulation and the reorganization of apico-basal polarity complexes<sup>10, 11</sup>, whereas another mitotic kinase, Plk1, couples the disassembly of planar cell polarity with mitotic division<sup>49</sup>. Furthermore, delocalization of apical polarity proteins, such as Par-6, aPKC, Baz and Crumbs, also occurs during symmetric cell division in



some epithelial cell types<sup>11, 29, 30, 44</sup>. A study in the *Drosophila* notum implicated lateral spreading of the apical proteins Par-6 and aPKC in the assembly of the isotropic actomyosin cortex that controls mitotic cell rounding<sup>30</sup>. Following our observation that Lgl cortical release occurs prior to the lateral spreading of Par-6 and aPKC<sup>11</sup>, it will be interesting to determine whether retaining Lgl at the cortex would also affect the cortical spreading of aPKC/Par-6, and subsequently their ability to drive cortical actin assembly.

The precise nature of Lgl regulation in distinct phosphorylation sites has yet to be elucidated. For instance, how site interdependence influences the importance of each serine along the cell cycle remains to be understood. One important conclusion that can be drawn from the analysis of single phosphorylatable mutants is that Lgl phosphorylation within the BH domain also positively controls Lgl activity in a site-specific manner. Future work will reveal how site specific phosphorylation number and order acts *in vivo* to control binding to the plasma membrane and cortical proteins. Furthermore, two other evolutionarily conserved serines within Lgl BH motif, and other phosphorylated sites detected by mass spectrometry using *Drosophila* embryos<sup>50</sup>, may provide alternative residues to fine-tune Lgl function. Given the involvement of Lgl in a myriad of cellular processes that control development, homeostasis and disease, understanding the conserved features of its regulation in multiple organisms will have important impact in the future of epithelial biology.

#### **Conflict of interest**

The authors declare no conflict of interest

#### **Acknowledgments**

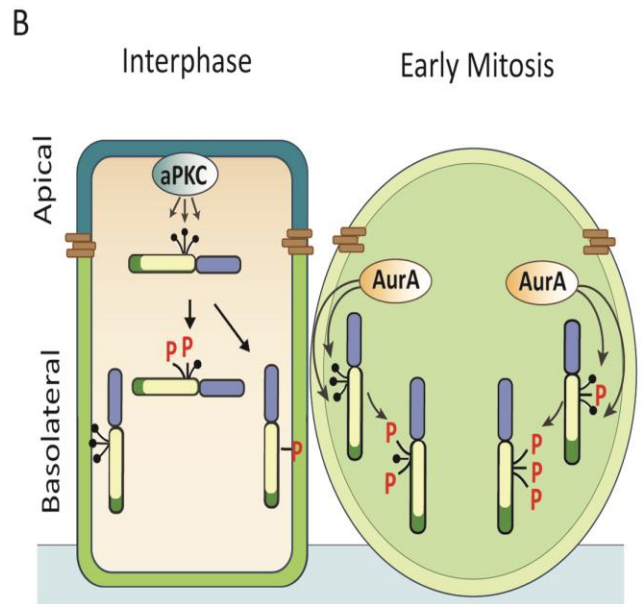
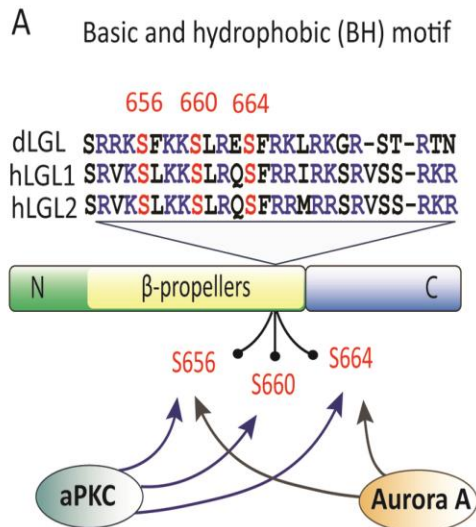
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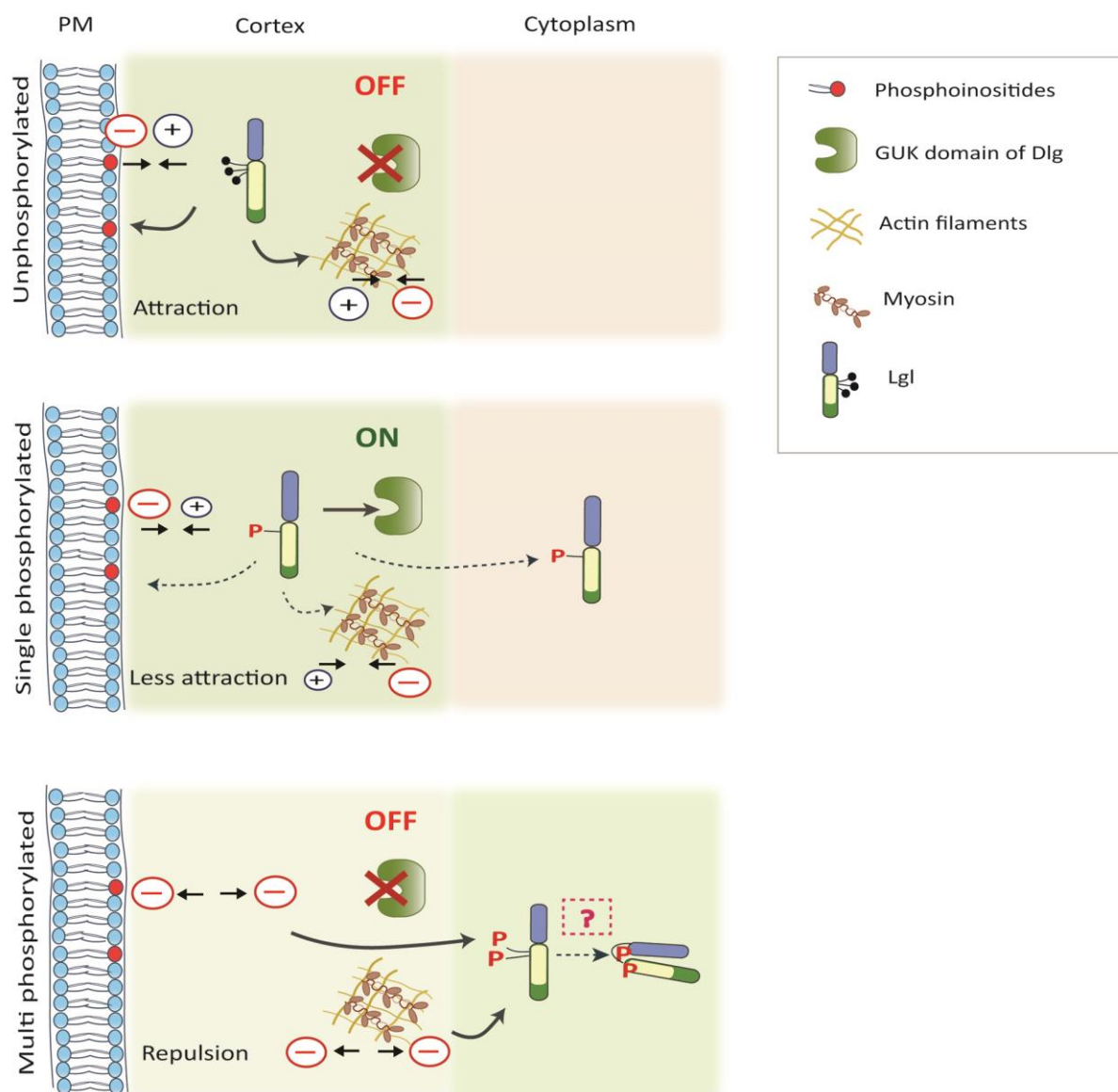
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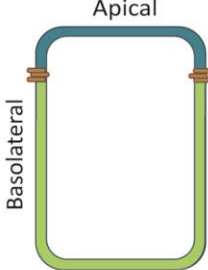
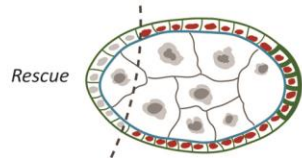
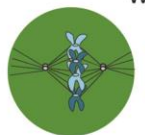
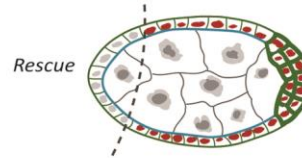
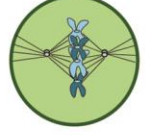
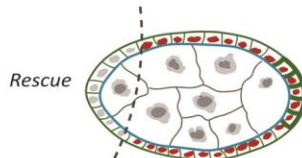

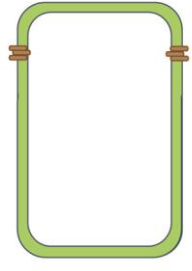
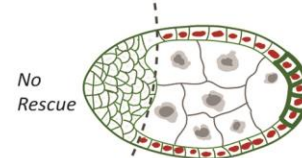
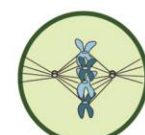
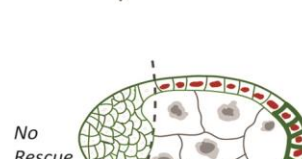

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**Figure 1: AurA and aPKC control Lgl localization during the cell cycle in epithelia.** (A) A conserved Basic and Hydrophobic (BH) domain of Lgl contains the three Serines controlled by aPKC and AurA. Positively charged residues are in blue. (B) Phosphorylation by aPKC on the apical domain controls basolateral restriction of Lgl during interphase. Multisite phosphorylation blocks membrane and cortical interactions, whereas 1P-Lgl may still interact with the plasma membrane, and may be required for interaction with some cortical binding partners. At mitosis onset, activation of Aurora A leads to multisite phosphorylation of Lgl inducing dissociation from the basolateral cortex.



**Figure 2: Model of phosphorylation as a molecular on/off switch of Lgl interactions.** The establishment of electrostatic interactions with phospholipids concentrates Lgl at plasma membrane (PM). Sequential phosphorylation progressively reduces the electrostatic interactions established by Lgl with cortical Myosin and phosphoinositides. In contrast, one phosphorylation seems to be required for the association of Lgl with the GUK domain of Dlg. Phosphorylation may therefore displace Lgl that was bound to the plasma membrane, allowing binding to specific cortical binding partners. Multisite phosphorylation blocks Dlg-Lgl interaction, possibly by further reducing the local concentration of Lgl or by inducing conformational/electrostatic changes that directly block Dlg-Lgl binding.

|                                      |          | Lgl localization in epithelial cells (Interphase)                                   | Rescue of <i>lgl</i> mutant and dominant effect of overexpression  | Localization in S2 cells (Mitosis)  |
|--------------------------------------|----------|---|--|---|
| Activity of Lgl mutants in epithelia | Higher   |   |  |   |
|                                      | Wt       |    | <br><i>lgl</i> mutant cells      Wild-type cells<br><i>Rescue</i> No dominant effect | <br>Wt   |
|                                      | AAS(664) |   | <br><i>Rescue</i> Dominant effect  | <br>AAS  |
|                                      | AS(660)A |   | <br><i>Rescue</i> No dominant effect   | <br>ASA  |
|                                      | Lower    |   |  |   |
|                                      | S(656)AA |  | <br>No Rescue      No dominant effect   | <br>SAA |
|                                      | AAA      |   | <br>No Rescue      No dominant effect  | <br>3A |

**Figure 3: Site-specific control of Lgl function and localization.** Schematic overview of the localization of singly phosphorylatable and non-phosphorylatable Lgl mutant forms during epithelial interphase and S2 cell mitosis<sup>11</sup>. Based on the ability of each mutant form to support epithelial polarity or to induce dominant basolateral activity upon strong overexpression, we speculate about the activity of each phosphorylatable site (left).